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TPC Proteins Are Phosphoinositide-Activated Sodium-Selective Ion Channels in Endosomes and Lysosomes

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SUMMARY

Mammalian two-pore channel proteins (TPC1, TPC2; *TPCN1*, *TPCN2*) encode ion channels in intracellular endosomes and lysosomes and were proposed to mediate endolysosomal calcium release triggered by the second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP). By directly recording TPCs in endolysosomes from wild-type and TPC double-knockout mice, here we show that, in contrast to previous conclusions, TPCs are in fact sodium-selective channels activated by $PI(3,5)P_2$ and are not activated by NAADP. Moreover, the primary endolysosomal ion is Na^+, not K^+, as had been previously assumed. These findings suggest that the organellar membrane potential may undergo large regulatory changes and may explain the specificity of $PI(3,5)P_2$ in regulating the fusogenic potential of intracellular organelles.

INTRODUCTION

Two-pore channel proteins (TPC1, TPC2; *TPCN1*, *TPCN2*) (Calcraft et al., 2009; Morgan et al., 2011) are localized in the intracellular endosomes and lysosomes (collectively endolysosomes), previously inaccessible to conventional patch-clamp assays. Consistent with this localization, human genetic studies have identified TPC2 as a regulator of pigmentation (Sulem et al., 2008), and a number of recent studies suggest that TPCs mediate Ca^{2+} release from endolysosomes in response to an elevation of the potent Ca^{2+}-mobilizing second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) (Brailoiu et al., 2009; Calcraft et al., 2009; Ruas et al., 2010; Zong et al., 2009) (but also see Guse, 2009). Unlike plasma-membrane-localized Na_V and Ca_V channels, the primary structures of TPCs contain two, instead of four, six-transmembrane domain repeats (Yu and Catterall, 2004). Like Na_V and Ca_V channels, they contain multiple positively charged amino acid residues in their voltage-sensor domains and negatively charged amino acid residues in their pore domains, but their intracellular localization has prevented characterization of basic channel properties such as selectivity and gating.

$PI(3,5)P_2$ is an endolysosome-specific phosphoinositide (PIP) of low abundance (Dove et al., 2009; Shen et al., 2011). Upon cellular stimulation, PIKfyve/Fab1 (PI 5-kinase) phosphorylates PI(3)P to increase $PI(3,5)P_2$ from low nanomolar to micromolar concentrations (Dove et al., 2009; Shen et al., 2011). Human mutations in $PI(3,5)P_2$-metabolizing enzymes and their regulators result in muscle and neurodegenerative diseases such as amyotrophic lateral sclerosis and Charcot-Marie-Tooth (CMT-4B, CMT-4J) disease (Chow et al., 2007). $PI(3,5)P_2$-deficient cells have enlarged endolysosomes/vacuoles, suggestive of impaired ion homeostasis and/or defective membrane trafficking (Chow et al., 2007; Dove et al., 2009; Kerr et al., 2010; Shen et al., 2011). We recently found that TRPML1 mediates $PI(3,5)P_2$-dependent Ca^{2+} release from endolysosomes (Dong et al., 2010a). However, $PI(3,5)P_2$ deficiency results in a much more severe phenotype than TRPML1 mutations, suggesting that there are additional $PI(3,5)P_2$ effectors (Shen et al., 2011). Here, we find by direct patch-clamp analysis of endolysosomal membranes that $PI(3,5)P_2$ specifically activates TPCs and, unexpectedly, that NAADP does not. TPC-mediated currents are selective for Na^+, which we demonstrate is the predominant cation in the lysosome. Thus, TPCs are intracellular Na^+-selective channels, suggesting a model for ion-channel control of endolysosomal fusion.

RESULTS

$PI(3,5)P_2$ Activation of a Large Endogenous Current in the Endolysosome

Cells were pretreated with vacuolin-1, a lipid-soluble polycyclic triazine (Huynh and Andrews, 2005) that can selectively increase the size of endosomes and lysosomes from less than 0.5 μm to

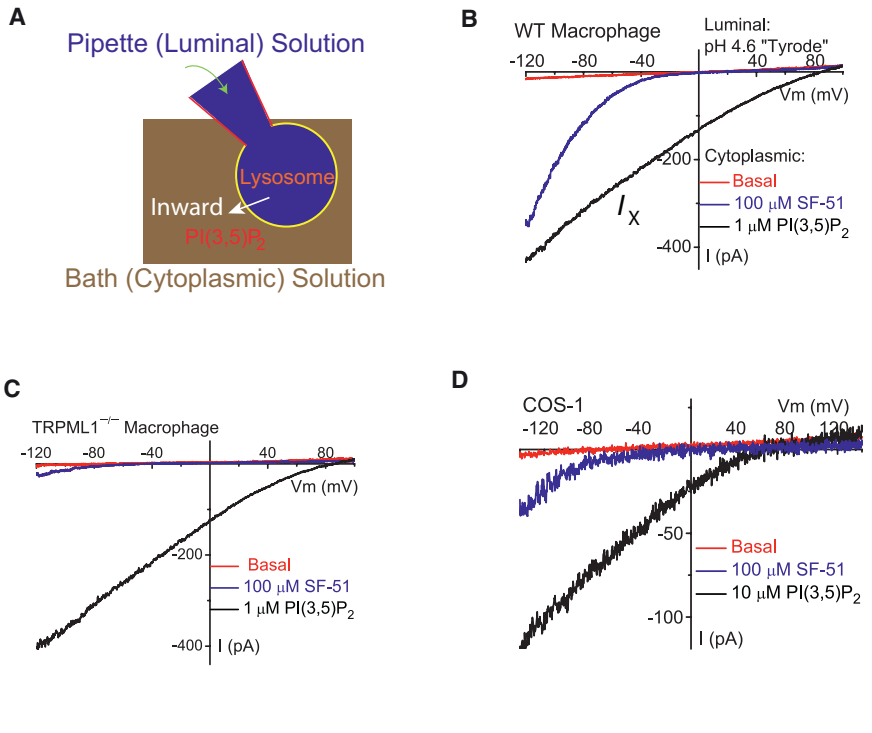

Figure 1. $PI(3,5)P_2$ Activates Endogenous TRPML1-Independent Inward Currents in Endolysosomes

(A) Illustration of the whole-endolysosome recording configuration. The pipette (luminal) solution was a standard external (modified Tyrode's) solution adjusted to pH 4.6 to mimic the acidic environment of the lysosomal lumen. The bath (internal/cytoplasmic) solution was a K^+-based internal solution (140 mM K-gluconate). Note that the inward current indicates cations flowing out of the endolysosome (arrow).

(B) Bath application of $PI(3,5)P_2$ (diC8, 1 μM) to the cytoplasmic side of enlarged endolysosomes/vacuoles isolated from vacuolin-treated WT primary macrophage cells activated whole-endolysosome currents (296 ± 28 pA/pF at −120mV, n = 24 vacuoles/endolysosomes) with positive E_{rev} (79 ± 2.4mV, n = 20). K^+-based cytoplasmic/bath solution contained 140 mM K^+, 4 mM Na^+, 2 mM Mg^{2+} (pH 7.2; free Ca^{2+} ~100 nM); luminal/pipette solution was a pH 4.6-modified Tyrode solution, which contained 145 mM Na^+, 5 mM K^+, 1 mM Mg^{2+}, 2 mM Ca^{2+} (pH 4.6); the equilibrium potential of Na^+ (E_{Na}) was estimated to be ~+90mV. Inwardly rectifying TRPML-like currents ($I_{TRPML\text{-}L}$) with E_{rev} = 3.7 ± 1.7mV (n = 20) were induced by a TRPML-specific small-molecule agonist (SF-51) in the same vacuoles (blue trace).

(C) $PI(3,5)P_2$ activated a current with a positive E_{rev} in an enlarged endolysosome/vacuole isolated from a *TRPML1*$^{-/-}$ primary macrophage cell.

(D) $PI(3,5)P_2$ (10 μM) activated whole-endolysosome current (from 30 to 420 pA measured at −120mV) with positive E_{rev} in enlarged endolysosomes/vacuoles isolated from nontransfected COS-1 cells. Note the small $I_{TRPML\text{-}L}$ (E_{rev} ~0mV) activated by SF-51 in the same vacuoles. Data are presented as mean ± SEM. See also Figure S1.

up to 5 μm (Dong et al., 2010a). The enlarged endolysosomes were manually isolated and then patch-clamped in the whole-endolysosome configuration (Figures 1A and S1 available online). We previously reported that TRPML1 was the primary $PI(3,5)P_2$-activated conductance (reversal potential, E_{rev} ~0 mV) in the endolysosomes of human fibroblasts (Dong et al., 2010a). However, in several other cell types, including skeletal muscles (data not shown) and macrophages, we observed that bath (cytoplasmic) application of diC8 $PI(3,5)P_2$ (abbreviated $PI(3,5)P_2$), a water-soluble analog of $PI(3,5)P_2$ (Dong et al., 2010a), activated a distinct whole-endolysosome conductance with an E_{rev} >+60mV (defined as I_X; Figure 1B). Strongly inwardly rectifying TRPML-like currents ($I_{TRPML\text{-}L}$; Dong et al., 2010a) were also present in macrophages, but $PI(3,5)P_2$-activated $I_{TRPML\text{-}L}$ was often masked by I_X due to its positive E_{rev}. $I_{TRPML\text{-}L}$ could be activated by SF-51 (100 μM; Grimm et al., 2010; Figure 1B). $I_{TRPML\text{-}L}$, but not I_X, was dramatically reduced in *TRPML1*$^{-/-}$ macrophages (Figure 1C). In 9 out of 23 enlarged endolysosomes isolated from nontransfected COS-1 cells, high concentrations of $PI(3,5)P_2$ (10 μM) activated I_X, which was distinct from $I_{TRPML\text{-}L}$ activated by 1 μM $PI(3,5)P_2$ (Dong et al., 2010a) or SF-51 (Figure 1D).

$PI(3,5)P_2$ Activates Recombinant TPC1 and TPC2 Channels in the Endolysosome

To search for the identity of the protein mediating I_X, a number of fluorescently tagged putative intracellular channels or transporter-like lysosomal-membrane proteins were transfected into COS-1 cells. As described below, endolysosomes from TPC1- and TPC2-transfected cells exhibit large I_X. The majority (>80%) of vacuolin-1-treated TPC2-positive vacuoles were Lamp-1$^+$ (Figure 2A), confirming that TPC2-positive vacuoles were enlarged late endosomes and lysosomes (LELs). In TPC2 (hTPC2)-positive enlarged LELs isolated from transfected COS-1 cells, little or no basal currents were detected in the whole-endolysosome configuration (Figure 2B). Bath application of $PI(3,5)P_2$ rapidly activated hTPC2-mediated currents (I_{TPC2}; E_{rev} = +83 ± 3mV; Figure 2B), but not those that expressed a mutant hTPC2 carrying a charge-reversal mutation in the putative pore domains (D276K; see Figures S2A and S2B); I_{TPC2} gradually declined upon the washout of $PI(3,5)P_2$ (Figure 2B) with variable time courses (depending on treatment time). The current-voltage (I-V) relationship and E_{rev} of I_{TPC2} are similar to the endogenous $PI(3,5)P_2$-activated I_X.

$PI(3,5)P_2$-dependent activation of I_{TPC2} was dose dependent (EC_{50} = 390 ± 94 nM; Figure 2C). I_{hTPC2} was inhibited >80% by the $PI(3,5)P_2$ chelators (Nilius et al., 2008; Suh and Hille, 2008) poly-L-lysine and anti-$PI(3,5)P_2$ antibody (Figures S2C and S2D). Other phosphoinositides, PI(3)P, PI(5)P, $PI(3,4)P_2$, $PI(4,5)P_2$, and $PI(3,4,5)P_3$, did not activate I_{TPC2} (10 μM; Figures 2D and S2E). Thus, $PI(3,5)P_2$ activated I_{TPC2} with striking specificity. In contrast, 1 μM $PI(3,5)P_2$ failed to activate the lysosome-localized (Lange et al., 2009) recombinant TRPM2 channel (Figure S2F) or modulate the endogenous outward currents that

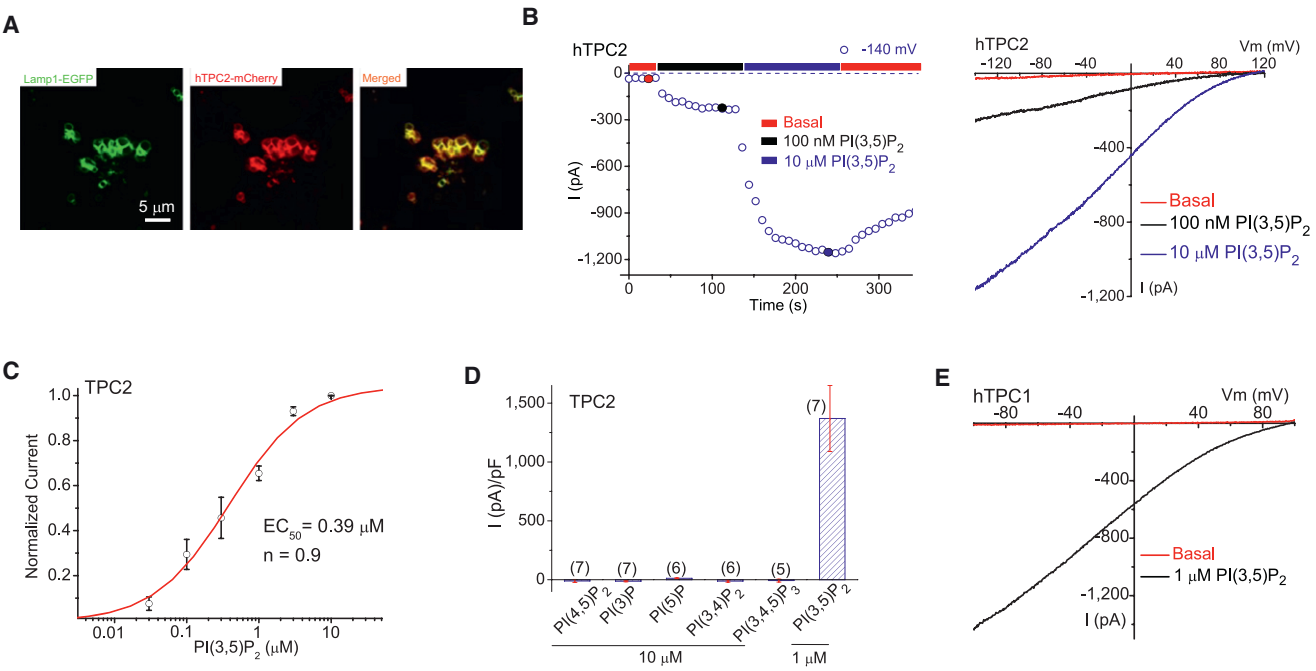

Figure 2. PI(3,5)P_2 Activates Recombinant TPCs in Endolysosomes

(A) TPC2 proteins are localized in Lamp-1-positive late endosomes and lysosomes in COS-1 cells that were transfected with TPC2 and Lamp-1 fusion proteins and treated with vacuolin-1.

(B) PI(3,5)P_2 activated a large whole-endolysosome current with E_{rev} >+80mV in an EGFP (hTPC2)-positive endolysosome isolated from an *hTPC2-EGFP*-transfected COS-1 cell. Whole-endolysosome currents were elicited by repeated voltage ramps (−140 to +140mV; 400 ms) with a 4 s interval between ramps; current amplitudes measured at −140mV were used to plot the time course of activation. Right: representative I-V traces of hTPC2-mediated whole-endolysosome currents (I_{hTPC2}) before (red; −20 ± 4 pA/pF at −120mV, n = 9) and after (black and blue) PI(3,5)P_2 bath application at three different time points, as indicated (left; red, blue, and black circles). Only a portion of the voltage protocol is shown; holding potential = 0mV.

(C) Dose dependence of PI(3,5)P_2-dependent activation (EC_{50} = 390 ± 94 nM, Hill slope [n] = 0.9, n = 13 vacuoles).

(D) Specific activation of TPC2 by PI(3,5)P_2 (in 1 μM), but not other PIPs (all in 10 μM). On average, I_{TPC2} in the presence of 1 μM PI(3,5)P_2 was 1,410 ± 360 pA/pF at −120mV (n = 7).

(E) Activation of I_{hTPC1} by 1 μM PI(3,5)P_2. Data are presented as mean ± SEM.

See also Figure S2.

were present in a subset of endolysosomes isolated from INS1 pancreatic β cells (Figure S2G). TPC1, also localized in the endolysosome (Calcraft et al., 2009) (but primarily in Lamp-1-negative compartments; Figure S2A), was also activated by PI(3,5)P_2 (Figure 2E).

TPC-Mediated Currents Are Na^+ Selective

The measured E_{rev} of I_{TPC} under standard recording conditions (with a low-pH-modified Tyrode's solution in the pipette/lumen and a K^+-based solution in the bath/cytosol) dictates that the channels are selective for Na^+, Ca^{2+}, or H^+, but not K^+. Increasing the luminal pH from 4.6 to 7.4 had minimal effects on I_{TPC2} (Figures 3A and S3A) and I_{TPC1}. Conversely, replacement of luminal cations (Na^+, K^+, Mg^{2+}, and Ca^{2+}) with $NMDG^+$ at pH 4.6 completely abolished inward I_{TPC2} (Figure S3B), suggesting that I_{TPC2} is impermeable to H^+ or $NMDG^+$. Under bi-ionic conditions (luminal Na^+, pH 7.4; cytoplasmic K^+), the E_{rev} of I_{TPC2} was +89 ± 5mV (n = 8; see Figure 3A). In contrast, under reversed bi-ionic conditions (luminal K^+, pH 7.4; cytoplasmic Na^+), the E_{rev} of I_{TPC2} was −68 ± 3mV (n = 5; see Figure 3B). These results indicated that I_{TPC2} was selective for Na^+ over K^+. Consistent with this conclusion, switching cytoplasmic K^+ to Na^+ in the presence of luminal Na^+ resulted in a leftward shift of the E_{rev} and the appearance of large outward currents (Figure 3A). Addition of 2 mM Ca^{2+} to the luminal side of the symmetric Na^+ solutions did not result in any significant change of the E_{rev} or the amplitude of the inward currents (see Figure S3C), suggesting that luminal Ca^{2+} contributed insignificantly to inward I_{TPC2}. Consistently, under bi-ionic conditions (luminal isotonic Ca^{2+}, pH 4.6 or 7.4; cytoplasmic Na^+), the E_{rev} of I_{TPC2} was −68 ± 2mV (n = 12; see Figure S3D), in dramatic contrast to the E_{rev} of I_{TRPML1} (+47 ± 2mV, n = 3; see Figure S3E). With cytoplasmic K^+, however, a small inward I_{TPC2} could be resolved with luminal isotonic Ca^{2+} (105 mM) but not $NMDG^+$ (Figures S3B and S3D), suggesting a very limited Ca^{2+} permeability for TPC2. By estimating the permeability ratios based on E_{rev} measurements, we determined the sequence of ion permeability or selectivity of I_{TPC2} as $Na^+ > Li^+ >> Ca^{2+} >> K^+ \sim Cs^+$ (Figures 3C and S3F). P_{Ca}/P_{Na} and P_K/P_{Na} were about 0.10 and 0.03, respectively, which are similar to the values for canonical Na_V channels (0.08–0.11) (Favre et al., 1996; Hille, 1972). Consistent with the low P_K/P_{Na}, with a mixture of K^+ and Na^+ at both luminal and

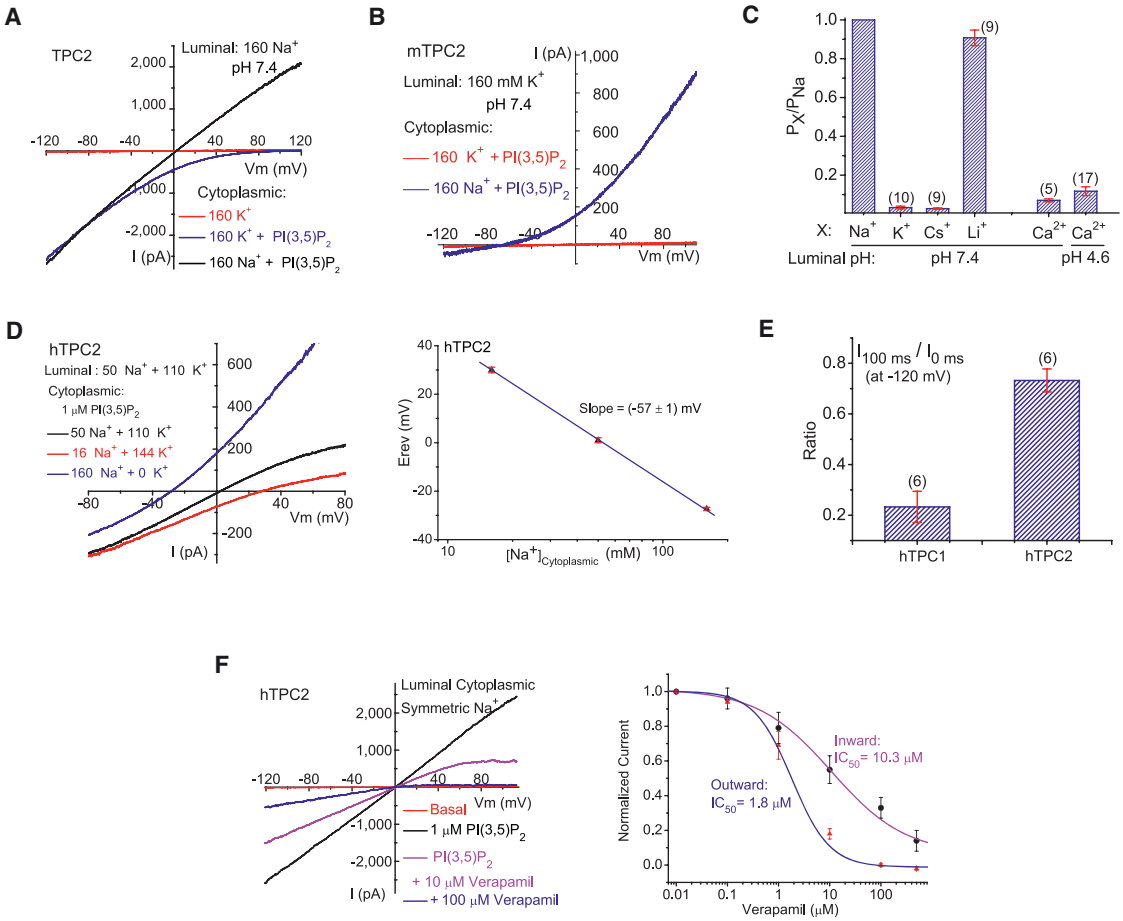

Figure 3. PI(3,5)P_2-Activated TPC Currents Are Na^+ Selective

(A) PI(3,5)P_2-activated I_{TPC2} (E_{rev} = +89 ± 5mV, n = 8) under bi-ionic conditions with luminal/pipette Na^+ and cytoplasmic/bath K^+. Large outward I_{hTPC2} was observed in cytoplasmic Na^+.

(B) I_{mTPC2} E_{rev} (−68 ± 3mV, n = 5) under bi-ionic conditions with luminal K^+ and cytoplasmic Na^+.

(C) Relative cationic permeability ratios of I_{hTPC2} based on E_{rev} measurement under bi-ionic conditions.

(D) Na^+ dependence of I_{hTPC2} E_{rev}. Left: I-V relations of I_{hTPC2} with cytoplasmic solutions containing various concentrations of Na^+ and K^+.

(E) Distinct inactivation kinetics of I_{hTPC2} and I_{hTPC1} at −120mV.

(F) Verapamil inhibited outward and inward I_{hTPC2} with different dose dependencies under luminal and cytoplasmic symmetric Na^+ (luminal pH 7.4). Data are presented as mean ± SEM.

See also Figure S3.

cytoplasmic sides, the Na^+ dependence of E_{rev} was fit with a Nernstian slope of 57mV per 10-fold change of $[Na^+]_{cyto}$ (Figure 3D). Taken together, these ion-substitution analyses demonstrate that TPC2 is a highly Na^+-selective channel in the endolysosome.

Because the S4 segments of TPC1 and TPC2 contain several positively charged amino acid residues, we investigated the voltage dependence of I_{TPC}. Unlike canonical Na_V and Ca_V channels, I_{TPC} was not directly activated by membrane depolarization. Instead, in response to a step-voltage protocol, PI(3,5)P_2-activated I_{TPC} inactivated at negative voltages (Figure S3G), with I_{TPC1} exhibiting faster inactivation than I_{TPC2} (Figure 3E). Inactivation recovered rapidly after a brief pulse to positive voltages (Figure S3H). Despite being Na^+ selective, I_{TPC2} was insensitive to the Na_V blocker tetrodotoxin (TTX) (Figure S3I) but was sensitive to low concentrations of the nonselective Ca_V blocker verapamil in a voltage-dependent manner (Figure 3F).

Na^+ Is the Major Cation in the Lysosome

The existence of Na^+-selective channels in the lysosome was unexpected because the lysosomal lumen, like the cytosol and the endoplasmic reticulum lumen (Morgan et al., 2011), has been presumed to contain high K^+ and low Na^+ (Morgan et al., 2011; Steinberg et al., 2010), suggesting the lack of a significant Na^+ or K^+ concentration gradient across the lysosomal membrane. To directly measure the ionic composition of the lysosome lumen, we enriched the lysosome fraction of HEK293T cells using density-gradient centrifugation (Dong et al., 2010a; Graves et al., 2008) (Figures 4A and S4A), and then determined the ratios of major cations (Na^+, K^+, Ca^{2+}, and

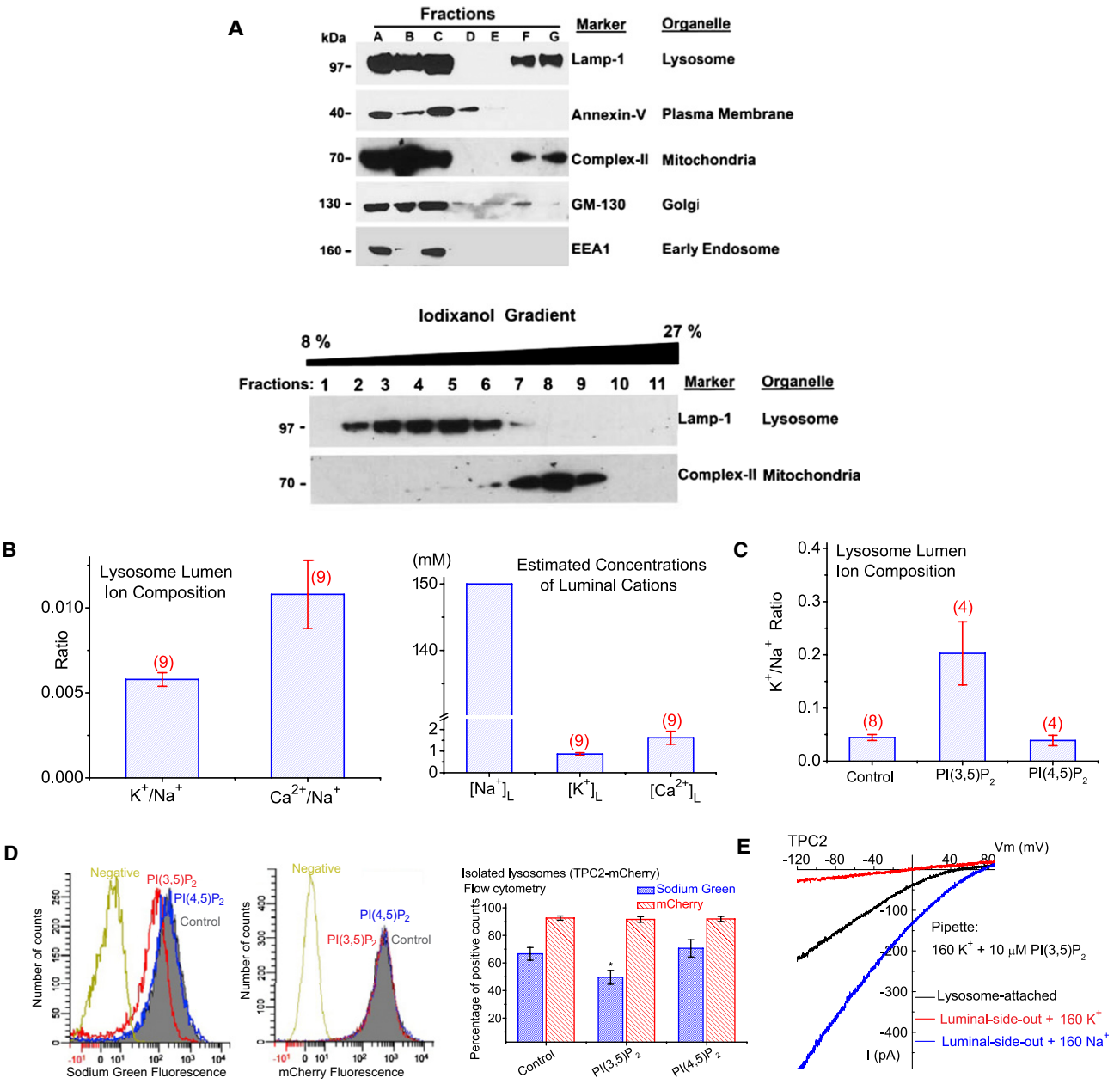

Figure 4. Na^+ Is the Major Cation in the Lysosome

(A) Western blotting was performed for each fraction (A–G) using various organelle markers: Lamp-1 for the lysosome; annexin-V for the plasma membrane; complex II for the mitochondria; GM-130 for the Golgi; EEA1 for the early endosome. The cellular fractionation protocol is described in Figure S4. Centrifugation of cell homogenate (fraction A) of HEK293T cells resulted in a pellet (fraction B) and a supernatant (fraction C). The supernatant was then layered over a discontinued gradient containing a cushion of 2.5 M sucrose and 18% Percoll in HM buffer. Further centrifugation of the gradient resulted in the light membrane fraction (fraction D), the medium membrane fraction (fraction E), and the heavy membrane fraction (fraction G). Lower: fraction G was then layered over a discontinuous iodixanol (8%–27%) gradient (fractions 1–11); western blotting was performed for each fraction using anti-Lamp-1 and anti-complex II.

(B) Ionic composition (Na^+, K^+, and Ca^{2+}) in the lysosomal lumen of HEK293T cells determined by ICP-MS. Right: estimated concentrations of Na^+, K^+, and Ca^{2+} in the lysosome lumen; estimates were based on the assumptions that the lysosome lumen is iso-osmotic relative to the cytosol and all the cations are osmotically active.

(C) Luminal K^+/Na^+ ratios were significantly increased for isolated lysosomes that were treated with $PI(3,5)P_2$ (20 μM) but not $PI(4,5)P_2$ (20 μM).

(D) Left: application of $PI(3,5)P_2$ (red) but not $PI(4,5)P_2$ (blue) to isolated lysosomes decreased sodium green but not mCherry fluorescence. Lysosomes were isolated from TPC2-mCherry-expressing HEK293 cells and loaded with sodium green dyes. "Control" and "negative" indicate fluorescence levels in isolated lysosomes with and without sodium green dye loading, respectively. Right: decreased sodium green fluorescence intensity (reflecting luminal Na^+ concentration) from $PI(3,5)P_2$-treated lysosomes; data are presented as the percentage of lysosomes that were sodium green positive. mCherry fluorescence remained constant upon $PI(3,5)P_2$/$PI(4,5)P_2$ application.

Mg^{2+}) using inductively coupled plasma mass spectrometry (ICP-MS) analysis. All centrifugation steps were performed at 4°C (1 hr in the Percoll density gradient + 2.5 hr in the iodixanol gradient; see Figure S4A). At this temperature, the rate of ion transport across the lysosomal membrane is expected to be extremely low. In addition, the sucrose-based homogenization buffer contains few ions. Thus, lysosomal ion transporters/exchangers are not likely to be operative. Hence, we presume that the lysosomal ion composition is largely maintained during the isolation procedure. Similar approaches have been used to determine ionic compositions in a number of intracellular organelles, including mitochondria and synaptic vesicles (Cohn et al., 1968; Schmidt et al., 1980). Although the absolute concentrations of ions could not be accurately measured due to the lack of information about lysosome volume, this approach allowed us to determine the relative abundance/ratios of the total but not free ions in the lumen. Interestingly, the K^+/Na^+ and Ca^{2+}/Na^+ ratios were only about 0.01 (Figure 4B), which was not significantly affected by the trace amount of ions in the buffer (Figure S4B). Similar results were obtained from human fibroblasts and mouse macrophages. Thus, Na^+ is the predominant cation in the lumen of the lysosome (estimated to be ~140–150 mM, assuming that its lumen is iso-osmotic relative to the cytosol and all the cations are osmotically active) (Figure 4B), indicating that in contrast to previous indirect measurements (Morgan et al., 2011; Steinberg et al., 2010), a large Na^+ concentration gradient is present across the lysosomal membrane.

To directly test whether the lysosomal lumen is a high-Na^+ compartment, isolated lysosomes were treated with TPC agonists. Application of $PI(3,5)P_2$, but not $PI(4,5)P_2$, significantly increased the K^+/Na^+ ratios (Figure 4C). Similarly, in isolated TPC2-mCherry lysosomes loaded with sodium green (Figure S4C), a Na^+-sensitive dye (Carrithers et al., 2007), $PI(3,5)P_2$, but not $PI(4,5)P_2$, application significantly decreased sodium green fluorescence (Figure 4D). These results suggest that sustained activation of TPCs may reduce luminal Na^+ content. Consistent with the lysosome being a high-Na^+ compartment rather than high-K^+, when $PI(3,5)P_2$ was included in the pipette solution, a large I_{TPC2} was observed under the lysosome-attached configuration (Figure 4E) in which the lysosomal content and hence the Na^+ gradient were maintained. Collectively, these results suggest that TPC-mediated Na^+ flux in response to a localized increase in $PI(3,5)P_2$ may rapidly depolarize endolysosomal membranes (luminal-side-positive ~+30–110mV at rest; Dong et al., 2010b; Morgan et al., 2011) and facilitate membrane fusion (Figure S4D). Consistently, TPC2-positive compartments were significantly enlarged in COS-1 cells transfected with WT, but not D276K mutant, hTPC2 (Figures S4E and S4E′), suggesting that TPC2-expressing endolysosomes might have increased fusogenic potentials.

TPC1 and TPC2 Underlie Endogenous TPC-like Currents in the Endolysosome

Mice lacking *TPC1* or *TPC2* were generated and crossed to make double-knockout ($TPC1^{-/-}/TPC2^{-/-}$) mice (Figure 5A). In our targeting strategy, the first exons of the *TPC1* and *TPC2* genes were deleted and the resulting recombinant transcripts failed to generate I_{TPC} (Figure S5A). In vacuoles isolated from $TPC1^{-/-}/TPC2^{-/-}$ primary macrophages, $PI(3,5)P_2$ activated $I_{TRPML\text{-}L}$ but Na^+-selective (I_{TPC}-like; $I_{TPC\text{-}L}$) currents were absent (Figure 5B). In contrast, $PI(3,5)P_2$ activated $I_{TPC\text{-}L}$ in the majority (>90%) of vacuoles in WT (Figure 5C) and $TRPML1^{-/-}$ (Figure 1C) macrophages. The current amplitudes of $I_{TRPML\text{-}L}$ were not significantly different in $TPC1^{-/-}/TPC2^{-/-}$ compared to WT macrophages (Figures 1B, 5B, and 5D), but were dramatically reduced in $TRPML1^{-/-}$ macrophages (Figures 1C and 5D). Although $I_{TRPML\text{-}L}$ and $I_{TPC\text{-}L}$ are both activated by $PI(3,5)P_2$, their I-V and E_{rev} differed significantly from each other. When we analyzed $PI(3,5)P_2$-activated currents at –30mV, large differences were noted between WT, $TRPML1^{-/-}$, and $TPC1^{-/-}/TPC2^{-/-}$ macrophages (Figure 5E). Consistently, the $PI(3,5)P_2$-activated current was selective for Na^+ over Ca^{2+} (Figure S5B). Collectively, these results suggest that $I_{TPC\text{-}L}$ is mediated by TPC2 and/or TPC1.

TPCs Are Not Activated by NAADP

Because TPC1 and TPC2 were reportedly activated by NAADP in endolysosomes (Brailoiu et al., 2009; Calcraft et al., 2009; Morgan et al., 2011; Ruas et al., 2010; Zong et al., 2009), we measured endolysosomal currents after direct application of NAADP. Surprisingly, in TPC2-positive enlarged vacuoles, no significant current activation was seen with varying concentrations of NAADP (Figures 6A and S6A). In contrast, $PI(3,5)P_2$ (10 μM) reliably (>90%) and robustly activated I_{TPC2} in the same vacuoles. NAADP (1–10 μM) also failed to modulate or desensitize I_{TPC2} that was activated by a low concentration of $PI(3,5)P_2$ (100 nM; Figure 6B). Similar results were seen with two NAADP analogs (4-methyl NAADP and 5-methyl NAADP) that induce Ca^{2+} release from sea urchin egg homogenates (Jain et al., 2010). I_{TPC1} was also insensitive to NAADP (Figure S6A). To exclude the possibility that NAADP responsiveness was impaired in vacuolin-enlarged endolysosomes, we also tested NAADP on surface-expressed mutant hTPC2 channels (i.e., hTPC2-$L^{11}L^{12}$/AA; see Brailoiu et al., 2010). However, plasma-membrane I_{TPC2}, which exhibited no notable difference in channel properties from lysosomal I_{TPC2}, was also insensitive to NAADP in inside-out patches and in the whole-cell configuration (Figures 6C and 6D). In contrast, NAADP (100 μM) activated NAADP-sensitive plasma-membrane I_{TRPM2} (see Tóth and Csanády, 2010; Figures S6B and S6C), demonstrating that NAADP was active.

Pancreatic β cells exhibit robust NAADP-mediated Ca^{2+} responses, and have been commonly used as a cellular model

(E) I_{TPC2} measured in lysosome-attached configuration. I_{TPC2} was activated in the lysosome-attached configuration with $PI(3,5)P_2$ (10 μM) in the K^+ pipette solution. I_{TPC2} was detected with luminal Na^+ but not K^+ upon excision into the luminal-side-out configuration. Note that the indicated voltages in the lysosome-attached configuration contained a contribution from the lysosomal-membrane potential, for which no accurate measurement is available. The smaller current amplitude seen in the lysosome-attached configuration might be due to luminal [Na^+] lower than 160 mM, relief from luminal inhibition, or both. Data are presented as mean ± SEM.

See also Figure S4.

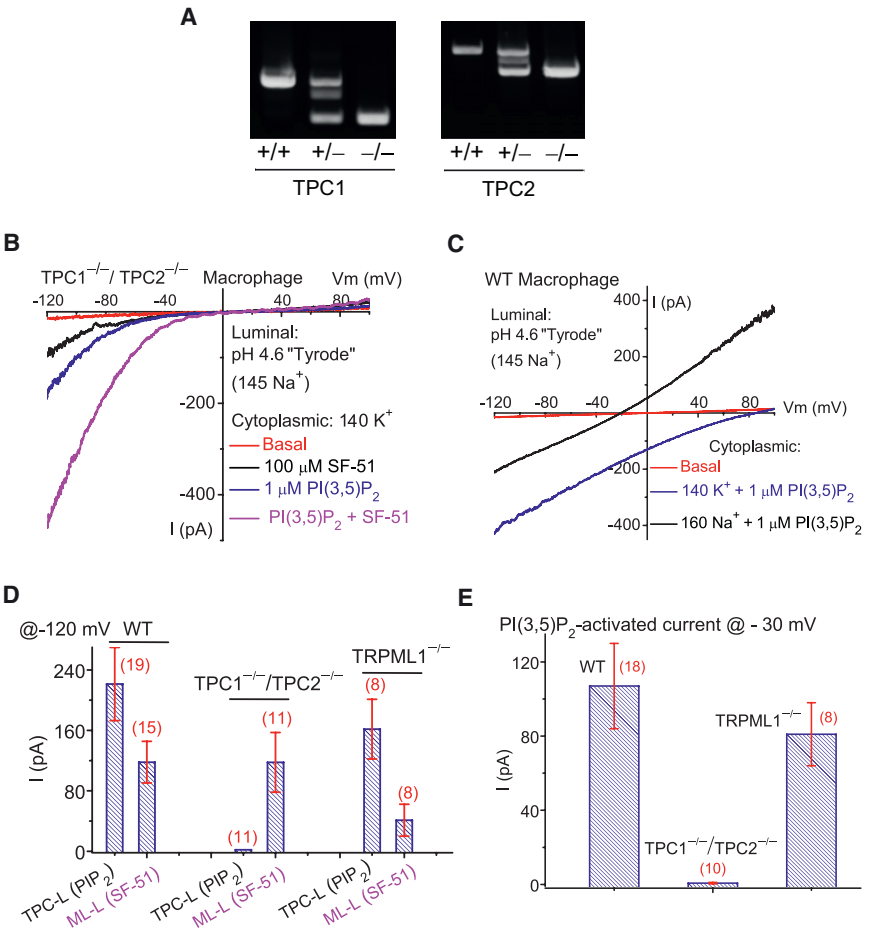

Figure 5. Genetic Inactivation of *TPC1* and *TPC2* Abolishes TPC Currents in the Endolysosome

(A) PCR genotyping of *TPC1* KO ($TPC1^{-/-}$) and *TPC2* KO ($TPC2^{-/-}$) mice.

(B) Lack of significant $PI(3,5)P_2$-activated TPC-like current ($I_{TPC\text{-}L}$) in vacuoles isolated from a $TPC1^{-/-}/TPC2^{-/-}$ mouse macrophage. Instead, in 15/15 vacuoles, $PI(3,5)P_2$ activated $I_{TRPML\text{-}L}$ that was further potentiated by SF-51.

(C) An endogenous $I_{TPC\text{-}L}$ activated by $PI(3,5)P_2$ (1 μM) in a vacuole isolated from a WT mouse macrophage cell. Switching the cytoplasmic solution from K^+ to Na^+ resulted in a leftward shift of E_{rev} and an increase of the current in the outward direction.

(D) Summary of $I_{TPC\text{-}L}$ and $I_{TRPML\text{-}L}$ in WT, $TPC1^{-/-}/TPC2^{-/-}$, and $TRPML1^{-/-}$ macrophages.

(E) Summary of $PI(3,5)P_2$-activated whole-endolysosome inward currents in WT, $TPC1^{-/-}/TPC2^{-/-}$, and $TRPML1^{-/-}$ macrophages at −30mV. I_{PIP2} was 107 ± 23 pA/pF (n = 18) and 0.7 ± 0.4 pA/pF (n = 10) for WT and $TPC1^{-/-}/TPC2^{-/-}$ macrophages, respectively. Data are presented as mean ± SEM.

See also Figure S5.

to study endogenous NAADP signaling (Morgan et al., 2011). In INS1 pancreatic β cell lines, intracellular perfusion with 100 nM NAADP in the whole-cell current-clamp configuration induced membrane depolarization and spike generation (Figure 7A). It has been reported that Ned-19, a membrane-permeable inhibitor of the NAADP receptor, completely inhibits NAADP- or glucose-induced Ca^{2+} responses at high micromolar concentrations (Naylor et al., 2009). However, Ned-19 had only a weak inhibitory effect on I_{hTPC2}, even at very high concentrations (1 mM; Figure S6D). Together with the fact that TPCs have limited Ca^{2+} permeability, these results suggest that TPCs do not contribute directly to NAADP-induced endolysosomal Ca^{2+} release.

TPC Currents Are Absent in Pancreatic β Cell Lines that Exhibit NAADP-Induced Lysosomal Ca^{2+} Release

Consistent with the results obtained from the pipette-dialysis experiments (Figure 7A), cell-permeant NAADP-AM (1–100 μM) (Parkesh et al., 2008) induced Ca^{2+} transients in INS1 (Figures 7B, 7C, S7A, and S7B) and MIN6 (see Figure S7D) pancreatic β cell lines in the absence or presence of external Ca^{2+}. NAADP-AM-induced Ca^{2+} responses in INS1 cells were abolished by the NAADP receptor blocker Ned-19 (Figures 7B, 7C, and S7A) or by pretreatment with bafilomycin A1, which inhibits V-ATPase to deplete acidic Ca^{2+} stores (Morgan et al., 2011) (Figures 7C and S7C). These results suggest that, consistent with previous studies, NAADP induces Ca^{2+} release from lysosomal stores. Surprisingly, no measurable NAADP-activated whole-endolysosomal current was seen in INS1 (Figures 7D and Figure S7E) or MIN6 (Figure S7F) cells. Furthermore, $PI(3,5)P_2$ (10 μM) activated $I_{TRPML\text{-}L}$ in 14/14 vacuoles, but I_X and $I_{TPC\text{-}L}$ were not detected (Figures 7D, S7E, and S7F). Collectively, NAADP-mediated responses appeared to be distinct from $I_{TPC\text{-}L}$ in pancreatic β cell lines, suggesting that TPCs do not contribute to the NAADP-mediated response.

TPC1 and TPC2 Are Not Required for NAADP- or Glucose-Induced Ca^{2+} Responses in Pancreatic Islets

Glucose induces robust Ca^{2+} responses in pancreatic β cells mediated via NAADP and its receptor localized in the endolysosome (Morgan et al., 2011; Naylor et al., 2009). However, in WT primary pancreatic β cells, we did not observe significant whole-endolysosome $I_{TPC\text{-}L}$ (Figure 7E). Glucose (5, 8, and 15 mM) induced significant increases of intracellular $[Ca^{2+}]$ (measured with Fura-2 Ca^{2+}-sensitive dyes) in pancreatic islets (Figure 7F), which were dramatically inhibited by Ned-19 (100 μM; Figure 7G), but the glucose-induced Ca^{2+} response was still largely intact in $TPC1^{-/-}/TPC2^{-/-}$ islets (Figures 7F and 7G). Finally, the NAADP-AM-induced Ca^{2+} response was not significantly reduced in $TPC1^{-/-}/TPC2^{-/-}$ pancreatic islets (Figure 7H). These results demonstrate that TPCs are not essential for NAADP- and glucose-induced Ca^{2+} responses in pancreatic β cells.

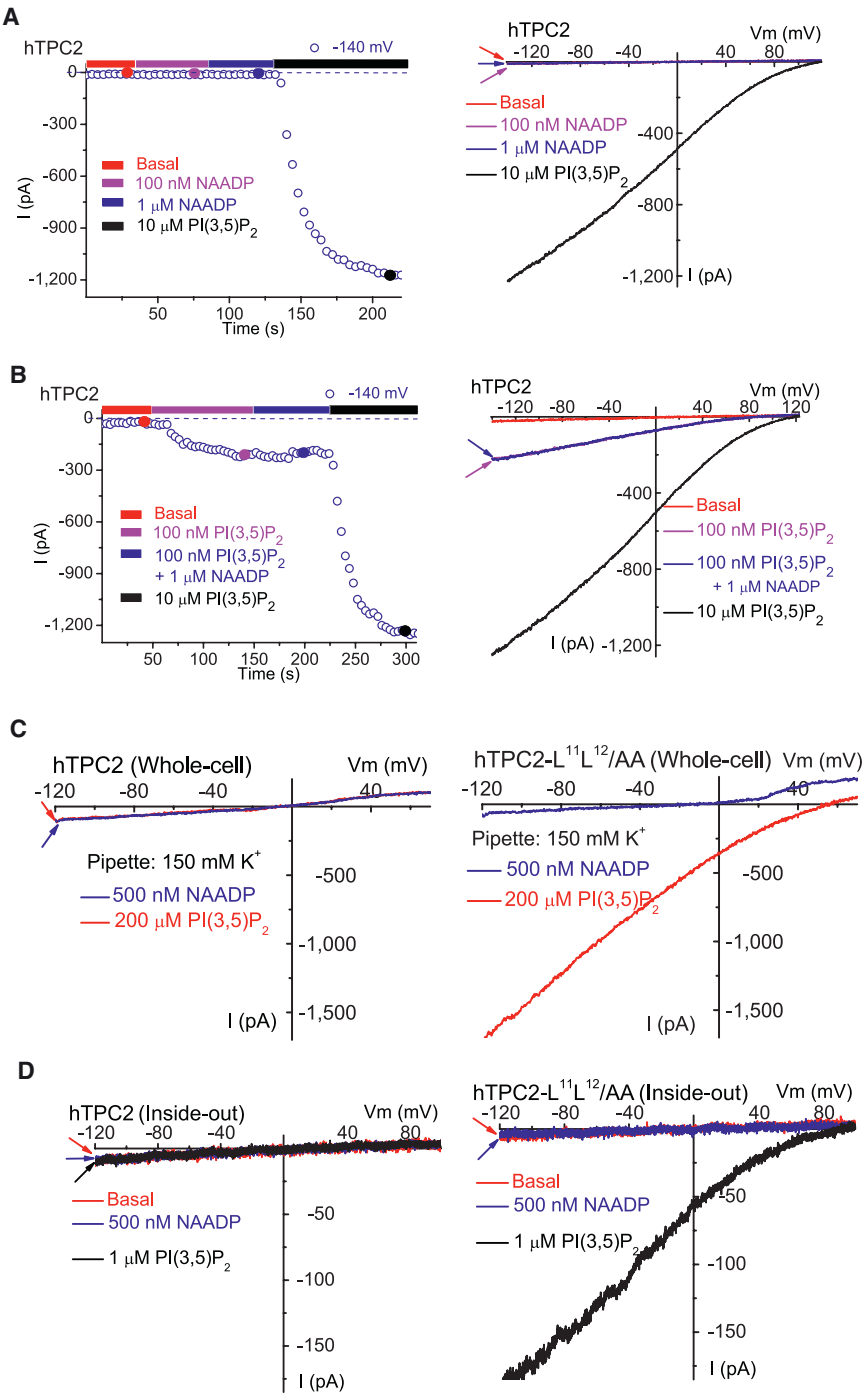

Figure 6. NAADP Does Not Activate TPCs

(A) NAADP (100 nM or 1 μM) failed to activate whole-endolysosome I_{hTPC2}. In contrast, PI(3,5)P_2 robustly activated I_{hTPC2} in the same vacuole. Right: representative I-V traces of whole-endolysosome currents at four different time points (indicated by color-coded circles) shown in the left panel.

(B) NAADP (1 μM) failed to modulate PI(3,5)P_2-activated I_{hTPC2}.

(C) Pipette dialysis of PI(3,5)P_2 (200 μM) or NAADP (500 nM) failed to elicit whole-cell current in HEK293T cells transfected with WT hTPC2. In contrast, pipette dialysis of PI(3,5)P_2 (200 μM), but not NAADP (500 nM), activated whole-cell I_{TPC2} in HEK293T cells transfected with a surface-expressed hTPC2 mutant (hTPC2-$L^{11}L^{12}$/AA).

(D) PI(3,5)P_2, but not NAADP, activated $I_{TPC2\text{-}LL\text{-}AA}$ in inside-out macropatches isolated from hTPC2-$L^{11}L^{12}$/AA -transfected HEK293T cells. Data are presented as mean ± SEM.

See also Figure S6.

DISCUSSION

TPCs have been reported to serve as the receptors (Brailoiu et al., 2009; Calcraft et al., 2009; Ruas et al., 2010; Zong et al., 2009) or coreceptors (Lin-Moshier et al., 2012) for NAADP, and NAADP-activated TPC currents were shown to be K^+ permeable (Pitt et al., 2010), Cs^+ permeable (Brailoiu et al., 2010), or Ca^{2+} selective (Schieder et al., 2010). These studies contrast drastically with our direct measurements of TPCs as NAADP-insensitive PI(3,5)P_2-activated Na^+-selective channels using whole-endolysosome patch-clamp recordings. Because NAADP-induced Ca^{2+} responses are robust in cells that lack $I_{TPC\text{-}L}$ and are largely intact in *TPC1*$^{-/-}$/*TPC2*$^{-/-}$ cells, and because our direct measurements of TPCs show that they are insensitive to NAADP, it should be clear that TPCs are not the NAADP receptor. Supporting this argument, recent studies using photoaffinity-labeled NAADP suggested that TPCs are unlikely to be genuine NAADP binding sites (Lin-Moshier et al., 2012). Thus, the reported effects of TPCs on NAADP-induced Ca^{2+} release from endolysosomes (Morgan et al., 2011) could arise from indirect mechanisms, for example, secondary to endolysosomal enlargement associated with TPC overexpression, or from the change of the local Na^+ gradient across the lysosomal membrane. Given that we have not been able to detect any NAADP-activated whole-endolysosome current, it is also possible that vacuolin treatment could impair the detection of NAADP-sensitive conductance. On the other hand, one cannot rule out that NAADP, like bafilomycin A1 and glycyl-L-phenylalanine 2-naphthylamide (GPN) (Morgan et al., 2011), might act via non-channel-mediated Ca^{2+} release mechanisms. NAADP might also target an unidentified anion conductance (low-Cl^- recording solutions are used in the current study) to induce lysosomal Ca^{2+} release. Finally, NAADP might also induce Ca^{2+} release from nonlysosomal Ca^{2+} stores, but

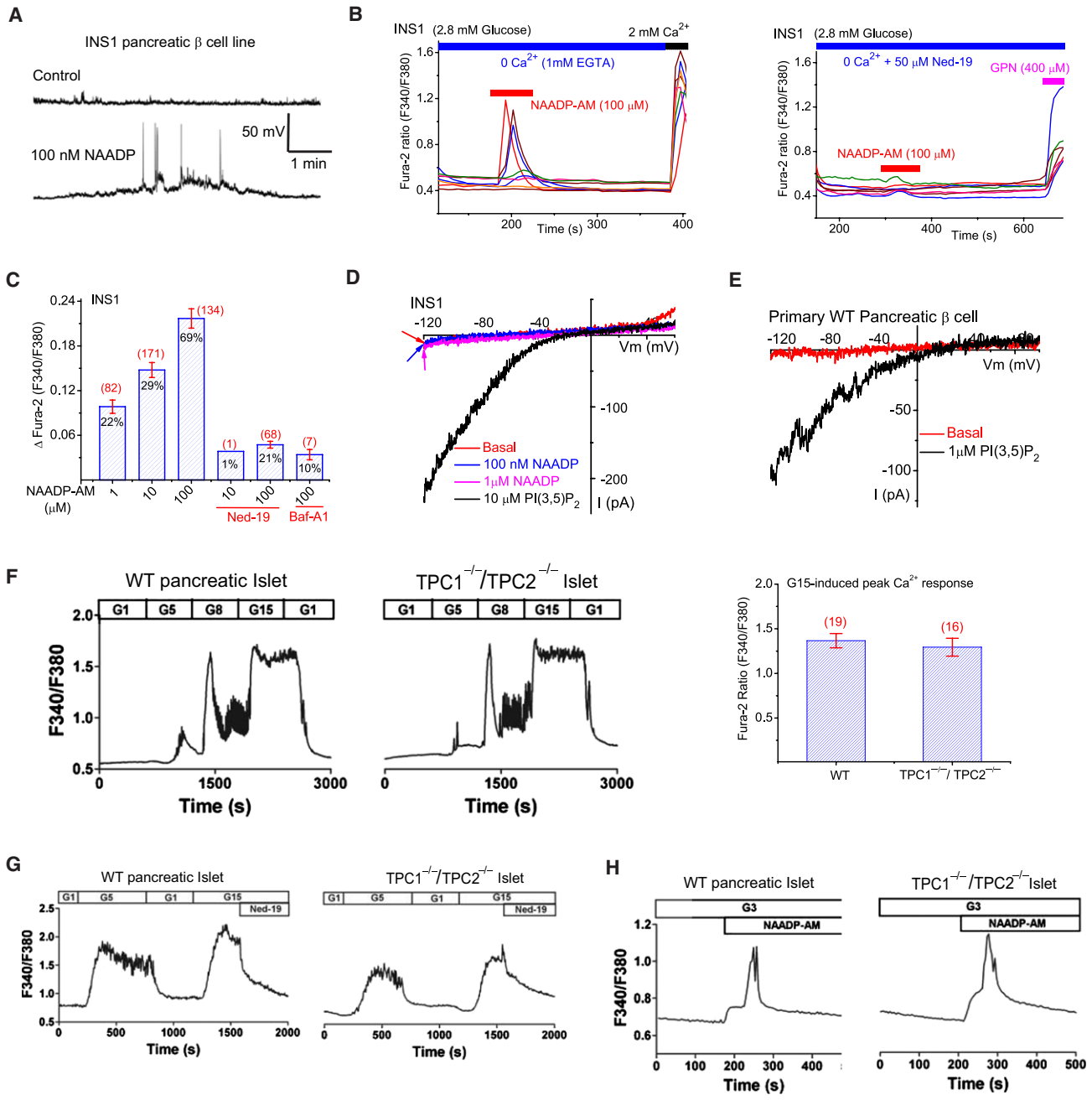

Figure 7. NAADP Induces Lysosomal Ca^{2+} Release in Cells that Lack TPC Currents or Proteins

(A) Pipette dialysis of NAADP (100 nM) induced membrane depolarization and spike generation in an INS1 pancreatic β cell line under the whole-cell current-clamp configuration.

(B) In the absence of external Ca^{2+} (free $[Ca^{2+}] < 10$ nM), NAADP-AM (100 μM) induced Ca^{2+} release measured with Fura-2 (F_{340}/F_{380}) ratios from intracellular stores in INS1 cells; Ned-19 (50 μM) abolished the majority of the NAADP-induced response.

(C) Average peak Ca^{2+} responses induced by NAADP-AM (1, 10, 100 μM) with and without Ned-19 and Baf-A1 (500 nM). Percentage (from a total of 100–400 cells) of responding (ΔFura-2 > 0.02) cells; the number of the responding cells is indicated.

(D) NAADP (100 nM or 1 μM) failed to induce measurable whole-endolysosomal current in a vacuole isolated from an INS1 cell; $PI(3,5)P_2$ activated large $I_{TRPML\text{-}L}$ (E_{rev} = 3.4 ± 3mV, n = 14; the ratio of current amplitudes at – 30mV versus −120mV was ~1%) in the same vacuole.

(E) $PI(3,5)P_2$ activated $I_{TRPML\text{-}L}$ in a vacuole from a WT primary pancreatic β cell.

(F) *TPC1*$^{-/-}$/*TPC2*$^{-/-}$ primary pancreatic islets exhibit normal concentration-dependent glucose-induced Ca^{2+} responses. Cytosolic $[Ca^{2+}]$ increased significantly in both WT and *TPC1*$^{-/-}$/*TPC2*$^{-/-}$ pancreatic islets in response to elevations in glucose concentration (in mM; 1, 5, 8, 15; G1, G5, G8, and G15) in the perfusion solution (2.5 mM Ca^{2+}). The traces shown are representative of 19 WT and 16 *TPC1*$^{-/-}$/*TPC2*$^{-/-}$ islets, respectively. Right: the average peak Ca^{2+} responses induced by 15 mM glucose (G15) in WT and *TPC1*$^{-/-}$/*TPC2*$^{-/-}$ pancreatic islets.

in a lysosome-dependent manner. Molecular identification of the NAADP-bound 22–23 kDa lysosomal proteins (Lin-Moshier et al., 2012) may help distinguish these possibilities.

The lysosomal lumen has been presumed to contain high K^+ and low Na^+ (Morgan et al., 2011; Steinberg et al., 2010), which would suggest the lack of a significant Na^+ or K^+ concentration gradient across the lysosomal membrane. These conclusions contrast directly with the high Na^+/low K^+ (like that of the extracellular media) we have found here using subcellular fractionation of organelles, which has been successfully applied to measure ionic compositions in a number of intracellular organelles, including mitochondria and synaptic vesicles (Cohn et al., 1968; Schmidt et al., 1980). Because the isolation procedures were performed at 4°C using a homogenization buffer that limits ion exchange, the lysosomal ion composition is presumed to be largely maintained. Indeed, lysosome fractions prepared using this protocol are of relatively normal size (lysosome swelling could be caused by the loss of luminal ions) and are functional (Graves et al., 2008; Radhakrishnan et al., 2008). Finally, the significant Na^+-selective current observed in the lysosome-attached configuration provides an independent verification that the lysosome lumen contains high concentrations of Na^+. It is worth mentioning that although a putative lysosomal K^+ release channel was proposed to provide counterion flux for lysosomal acidification (Steinberg et al., 2010), such a scenario is unlikely to occur due to the high cytosolic K^+ and hence the low or opposite electrochemical gradient of K^+ across the lysosome membrane. Instead, the existence of a large Na^+ gradient and lysosomal Na^+ channels is more likely to fulfill this function (counterion flux).

What is the purpose of Na^+-selective, $PI(3,5)P_2$-activated TPC channels? Increases in $PI(3,5)P_2$ will allow Na^+ to move down its concentration gradient, rapidly reducing and reversing (Figure S4D) the endolysosomal potential, which is presumed to be luminal-side positive at rest (estimated to be +30 to +110mV) (Dong et al., 2010b). In model membranes, it has been demonstrated that Na^+ and K^+ exhibit differential effects on membrane curvature (Kraayenhof et al., 1996). Whereas oppositely charged lipid bilayers tend to fuse (Anzai et al., 1993), Na^+ influx into the cytoplasm reportedly affects membrane fusion during exocytosis (Parnas et al., 2000). Thus, TPC-mediated Na^+ flux in response to a localized increase in $PI(3,5)P_2$ may rapidly depolarize endolysosomal membranes and promote fusion (Figure S4D). Consistent with a previous study (Ruas et al., 2010), we found that TPC overexpression results in enlarged endolysosomes; this might be caused by enhanced endolysosomal fusion, decreased fission, or both. However, unlike TRPML1 (Shen et al., 2011), TPCs are not expressed in every cell type, suggesting that their role in membrane trafficking is more specific. Furthermore, because membrane fusion could occur even in in vitro reconstitution systems, neither TPCs nor TRPMLs are necessarily required as direct participants in the basic membrane fusion machinery (Shen et al., 2011). However, they may regulate the direction and specificity of lysosomal trafficking in vivo. Indeed, lysosomal trafficking is significantly delayed, although not blocked, in cells lacking TRPML1 (Shen et al., 2011). Future research may reveal the relative importance of Na^+ versus Ca^{2+} and TRPMLs versus TPCs in spatial and temporal regulation of lysosomal trafficking. Finally, in addition to defining organelle specificity and determining the fusogenic potential of endolysosomes, the proposed cellular functions of $PI(3,5)P_2$ also include regulating endolysosomal ion homeostasis, especially H^+ homeostasis (Kerr et al., 2010; Shen et al., 2011). The proposed role of a putative monovalent cation (K^+ or Na^+) conductance in lysosomal acidification (Steinberg et al., 2010), together with our demonstration of a large Na^+ gradient across the endolysosomal membrane, suggests that $PI(3,5)P_2$-sensitive Na^+-permeable TPCs, but not K^+ release channels (see above), may participate in endolysosomal pH regulation in a transient and localized manner. In addition, rapid changes in Na^+ content will drive Na^+/H^+ exchangers in the organelle membrane, thus changing organellar pH. The exact sequence of events will depend on expanding our knowledge of transporters in endolysosomal membranes and finding more accurate methods to measure endolysosomal potentials in intact cells.

EXPERIMENTAL PROCEDURES

Targeted Deletion of *TPC1* and *TPC2* in Mice

TPC1 and *TPC2* double-knockout mice were generated as described in Extended Experimental Procedures. Animals were used according to approved animal protocols and institutional animal care guidelines at the University of Michigan.

Endolysosomal Electrophysiology

Endolysosomal electrophysiology was performed in isolated enlarged endolysosomes using a modified patch-clamp method (Dong et al., 2010a). Cells were treated with 1 μM vacuolin-1, a lipid-soluble polycyclic triazine that can selectively increase the size of endosomes and lysosomes (Huynh and Andrews, 2005), for at least 1 hr or up to 12 hr. Large vacuoles (up to 5 μm; capacitance = 1.1 ± 0.1 pF, n = 29 vacuoles) were observed in most vacuolin-treated cells. Occasionally, enlarged vacuoles were also seen in nontreated cells; no significant difference in TPC properties were seen for enlarged vacuoles obtained with or without vacuolin-1 treatment. Whole-endolysosome recordings were performed on manually isolated enlarged endolysosomes (Dong et al., 2010a). In brief, a patch pipette was pressed against a cell and quickly pulled away to slice the cell membrane. Enlarged endolysosomes were released into a dish and identified by monitoring EGFP-TPC1/2, mCherry-TPC1/2, or EGFP-Lamp-1/mCherry-Lamp-1 fluorescence. After formation of a gigaseal between the patch pipette and the enlarged endolysosome, capacitance transients were compensated. Voltage steps of several hundred millivolts with millisecond duration were then applied to break into the vacuolar membrane (see Figure S1). The whole-endolysosome

(G) Glucose-induced Ca^{2+} responses in WT and *TPC1*$^{-/-}$/*TPC2*$^{-/-}$ pancreatic islets were inhibited by Ned-19 (100 μM). The traces shown are representative of the results obtained in seven WT and seven *TPC1*$^{-/-}$/*TPC2*$^{-/-}$ islets, respectively.
(H) NAADP-AM (200 μM) induced biphasic Ca^{2+} increases in the presence of 3 mM glucose (Yamasaki et al., 2004) in both WT and *TPC1*$^{-/-}$/*TPC2*$^{-/-}$ islets. Traces shown are representative of the results obtained in two WT and three *TPC1*$^{-/-}$/*TPC2*$^{-/-}$ islets, respectively. Data are presented as mean ± SEM.
See also Figure S7.

configuration was verified by the reappearance of capacitance transients after break-in (see Figure S1).

Unless otherwise stated, bath (internal/cytoplasmic) solution contained 140 mM K-gluconate, 4 mM NaCl, 1 mM EGTA, 2 mM $MgCl_2$, 0.39 mM $CaCl_2$, 20 mM HEPES (pH was adjusted with KOH to 7.2; free $[Ca^{2+}]_i$ ~100 nM). In a subset of experiments, 2 mM Na_2-ATP and 0.1 mM GTP were added to the bath solution and the pH was readjusted. The pipette (luminal) solution was standard extracellular solution (modified Tyrode's: 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM MES, 10 mM glucose; the pH was adjusted with NaOH to pH 4.6). In a subset of experiments, a low-Cl^- pipette solution containing Na-gluconate replaced NaCl. All bath solutions were applied via a perfusion system that allowed us to achieve complete solution exchange within a few seconds. Data were collected using an Axopatch 2A patch-clamp amplifier, Digidata 1440, and pClamp 10.2 software (Axon Instruments). Whole-endolysosome currents were digitized at 10 kHz and filtered at 2 kHz. All experiments were conducted at room temperature (21°C –23°C), and all recordings were analyzed with pClamp 10.2 and Origin 8.0 (OriginLab). All PIPs were from A.G. Scientific; water-soluble diC8-PIPs, prepared in high-concentration stock solutions, were dissolved in the bath solutions and delivered via the perfusion system at low concentrations (0.1–1 μM) and direct bath application at higher concentrations (10 μM). NAADP, 4-methyl NAADP, and 5-methyl NAADP were from Tocris Bioscience, Sigma, or kindly provided by James Slama (Jain et al., 2010). The permeability to cations (relative to P_{Na}) was estimated based on E_{rev} measurement under bi-ionic conditions as described in Extended Experimental Procedures.

Ca^{2+} Imaging

Ca^{2+} imaging was performed using an EasyRatioPro system (Photon Technology International) as described in Extended Experimental Procedures.

Lysosome Isolation by Subcellular Fractionation

Lysosomes were isolated as described previously (Dong et al., 2010a; Graves et al., 2008). Briefly, cell lysates were obtained by Dounce homogenization in a homogenizing buffer (HM buffer; 0.25 M sucrose, 1 mM EDTA, 10 mM HEPES; pH 7.0), and then centrifuged at 1,900 × *g* (4, 200 rpm) at 4°C for 10 min to remove the nuclei and intact cells. Postnuclear supernatants then underwent ultracentrifugation through a Percoll density gradient using a Beckman L8-70 ultracentrifuge. An ultracentrifuge tube was layered with 2.5 M sucrose, 18% Percoll in HM buffer. Centrifugation speed was 67,200 × *g* (14,000 rpm) at 4°C for 1 hr using a Beckman Coulter 70.1 Ti rotor. Samples were fractionated into light, medium, and heavy membrane fractions. Heavy membrane fractions contained concentrated bands of cellular organelles and were further layered over a discontinuous iodixanol gradient. The iodixanol gradient was generated by mixing iodixanol in HM buffer with 2.5 M glucose (in v/v; 27%, 22.5%, 19%, 16%, 12%, and 8%); the osmolarity of all solutions was ~300 mOsm. After centrifugation at 4°C for 2.5 hr, the sample was divided into ten fractions (0.5 ml each) for biochemical and atomic absorption analyses. Note that the ionic composition of the lysosome was largely maintained due to the low rate of ion transport across the lysosomal membrane at 4°C. Antibodies used for western blots were anti-Lamp-1 (Iowa Hybridoma Bank), 1:5,000 dilution; anti-Annexin V (Abcam), 1:2,000 dilution; anti-GM130 (Abcam), 1:2,000 dilution; anti-EEA1 (Santa Cruz Biotechnology), 1:500 dilution; anti-Complex II (Abcam), 1:5,000 dilution; and anti-GFP (Covance), 1:5,000 dilution.

Inductively Coupled Plasma Mass Spectrometry

Lysosomal fractions were prepared for atomic absorption by diluting the samples in a 1/1 ratio with concentrated nitric acid. After digestion (10 min, 60°C), the ionic composition was measured using a Thermo Scientific Finnigan Element inductively coupled with a plasma high-resolution mass spectrometer (Séby et al., 2003).

Data Analysis

Data are presented as mean ± SEM. Statistical comparisons were made using ANOVA. A p value <0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.08.036.

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